REMARKS

35 U.S.C. §112, second paragraph

The Examiner has rejected Claims 3, 4, 9-11, and 13-15 under 35 U.S.C. §112, second paragraph for failing to particularly point out and distinctly claim the subject matter which Applicants' regard as the invention. According to the Examiner, the term "hot start DNA polymerase" is vague and indefinite as,

> "The term 'hot start' is a term of art and may be interpreted as reading on a variety of methods of supplementing or enhancing amplification specificity, including physical partitioning of reagents, modification of polymerase enzymes, or the inclusion of a reversibly inhibitory antibody directed against the DNA polymerase." (Office Action, page 3.)

Applicants assert that the term "hot start DNA polymerase" as used in the present application and in the claims is a well defined term of art, the scope and meaning of which are readily understood by the skilled practitioner.

For example, to demonstrate that the scope and meaning of "hot start DNA polymerase" was a well-defined term at the time the present application was filed, pursuant to MPEP 609.05(c), Applicants submit herewith U.S. Pat. No. 6,312,929 filed December 22, 2000, entitled "Compositions And Methods Enabling A Totally Internally Controlled Amplification Reaction" (attached at Tab A). The '929 patent sets forth the art-recognized definition, at the time the present application was filed, for the term "hot start DNA polymerase" as follows:

> In some embodiments, a 'hot start' polymerase can be used to prevent extension of mispriming events as the temperature of a reaction initially increases. Hot starts are particularly useful in the context of multiplex PCR. Hot start polymerases can have, for example, heat labile adducts requiring a heat activation step (typically 95°C for approximately 10-15 minutes) or can have an antibody associated with the polymerase to prevent activation." (See, '929 patent, column 11, lines 26-33.)

See also, for example, International application PCT/GB01/02265 (published as WO 01/92569), attached at Tab B:

> "The polymerase may be a DNA polymerase and may be a thermally stable polymerase, e.g. Taq polymerase. There is however a deficiency with Taq in that it becomes active (though at low efficiency) at relatively low temperatures. As a result, mis-primed reactions can occur at these low temperatures before the PCR begins. . . . It is therefore preferred that the polymerase in the formulation of the invention is a "hot-start" polymerase. "Hot-start" polymerases are known in the art and are such that a heating step is required to activate the polymerase (which has typically been inactivated with an antibody). The "hot-start" polymerase should be one for which the "means" of inactivation of the enzyme (e.g. an antibody) must be able to withstand the drying/rehydration procedure. The advantage that the use of such an enzyme confers is that the re-hydration of the dried reagent composition can occur at ambient temperature without initiating potentially ruinous side reactions prior to heating the sample and cooling to annealing temperature at which only desired reactions can occur." (WO 01/92569 at pages 5-6.)

Therefore, "hot start DNA polymerase" as used in the present application is directed to a DNA polymerase modified to exhibit essentially no polymerase activity at lower temperature (ambient temperatures), i.e., to prevent non-specific primer binding and extension, but which is activated after exposure to high temperatures (e.g., 95°C for 10-15 min.). Hot start DNA polymerases are products known in the art and are commercially available (see, e.g., HotStarTaq® DNA polymerase, Qiagen GmbH, Qiagen Product Guide 2002, p. 37 attached at Tab C).

The present application also provides a particular reference for suitable "hot start DNA polymerases" by specific reference to U.S. Pat. No. 6,183,998 entitled "Method For Reversible Modification Of Thermostable Enzymes", which is incorporated by reference into the present specification (see, page 27, line 9). A copy is attached at Tab D.

The '998 patent discloses aldehyde-modified DNA polymerases, which are preferred as they overcome some of the disadvantages of other available hot start enzymes. As disclosed in the '998 patent:

> "To overcome difficulties related to non-specific amplification products caused by the extension of mis-primed oligonucleotides during the reaction set-up or the initial heating phase of PCR, an essential PCR component such as the oligonucleotide primers, nucleotide triphosphates, magnesium ions or thermostable nucleic acid polymerase could be added only at higher temperatures, thereby reducing the probability of having non-

specific hybridization or extending mis-primed oligonucleotides. This technique is commonly known as "**hot-start** PCR", or more specifically "**manual hot-start** PCR". ('998 patent at paragraph bridging cols. 1-2, emphasis added.)

* * *

"Another method of reducing formation of extension products from mis-primed oligonucleotides during the reaction set-up is a reversible non-covalent modification of the nucleic acid polymerase. U.S. Pat. No. 5,338,671 discloses the use of antibodies specific for the nucleic acid polymerase to inhibit the polymerase's activity. Pre-mixing of nucleic acid polymerase and polymerase-specific antibodies results in the formation of an antibody-polymerase complex. Under these conditions substantially no oligonucleotide extension activity can be detected. At elevated temperatures, the antibody dissociates from the complex, thus releasing the nucleic acid polymerase, which can then function in DNA synthesis during the Polymerase Chain Reaction. However, this method carries the risk of contamination due to an increased number of handling steps and the possible presence of residual nucleic acids derived from the antibody preparation. Another method to reduce non-specific amplification products involves the use of a chemically modified thermostable DNA polymerase that becomes active only after incubation of the DNA polymerase for a certain period of time at elevated temperature, thus preventing production of non-specific DNA synthesis products during reaction set-up and the initial heating phase of PCR. U.S. Pat. No. 5,677,152 and corresponding European patent publication EP 0 771 870 A1 describe a method for amplification of a target nucleic acid using a thermostable polymerase reversibly inactivated using a dicarboxylic acid anhydride. ('998 patent, col. 2, lines 30-56.)

* * *

". . . The modified thermostable DNA polymerases according to the present invention exhibit stable, reduced enzyme activity at temperatures lower than about 50° C., and thus the presently disclosed reversibly inactivated enzymes show advantages over, e.g., a DNA polymerase treated as described in European patent publication EP 0 771 870 A1, as demonstrated in Examples 9, 12 and 13 below, due to the conditions necessary for reactivation . . . ('998 patent at col. 4, lines 60-67.)

* * *

". . . The modified thermostable enzyme will have essentially no primer extension activity at room temperature and will not recover significant activity until exposed to elevated

> temperatures. Below about 50°C., however, exposure must be so prolonged (greater than 1 hour) in order to reactivate the enzymes that the enzyme modification is viewed as being reversible only at high temperatures (above 50°C.). In a preferred embodiment, for example, a modified Taq DNA polymerase treated with formaldehyde according to the present invention has essentially no primer extension activity at the conditions of maximal primer extension activity (i.e., between 60°C. to 75°C.), as shown in Example 6; and the modified thermostable enzyme appears to be further stabilized in comparison with Taq DNA polymerase treated with anhydride." ('998 patent, paragraph bridging cols. 5-6.)

It is seen that the term "a hot start DNA polymerase" is well understood by those skilled in the art and is a term sufficiently precise to inform those skilled in the art how to make and use the present invention, fully satisfying the requirements of 35 U.S.C. §112.

In view of the foregoing remarks, reconsideration of this objection and allowance of Claims 3, 4, 9-11, and 13-15 are respectfully requested.

35 U.S.C. 112, second paragraph

The Examiner has rejected Claims 9-11 and 13-15 as vague and indefinite as the reference to the molecular weight of the volume exclusion agents disclosed therein, i.e., polyethylene glycol (Claims 9-11) and dextran (Claims 13-15) does not include the units of weight measurement therefor. Applicants have amended Claims 9-11 and 13-15 to specify that the molecular weight units of the polyethylene glycol and dextran are expressed in daltons. Support for the amendments may be found in the specification on page 17, lines 4-7. No new matter is added by the amendments to Claims 9-11 and 13-15. Entry of the amendments and allowance of the claims are respectfully requested.

35 U.S.C. §103(a)

The Examiner has rejected Claims 1-14 and 16 under 35 U.S.C. §103 as being unpatentable over Backus et al., U.S. Pat. No. 5,705,366[1], in view of Bustin, *Journal of Molecular Endocrinology*, 25: 169-193 (2000).

According to the Examiner, with respect to Backus,

[1] Applicants note that the Examiner cites U.S. Pat. No. 5,703,366 throughout the Office Action. However, U.S. Pat. No. 5,703,366 is issued to Sting et al. and is entitled "Optical Sensing With Crystal Assembly Sensing Tip".

> "Backus discloses a method of amplification of multiple target nucleic acids in the presence of a nonionic, polymeric volume exclusion agent." (See, Office Action, page 4.)

With respect to Bustin, the Examiner states,

> ". . . Bustin teaches the coamplification of nucleic acids which are present at comparable copy numbers, wherein the maximum difference between the lowest and highest copy number is 10-fold (p. 182, col. 1, 2nd paragraph, where the normalization of quantification of a target nucleic acid is accomplished through co-amplification of an internal control target sequence, referred to as an endogenous control. It is also noted that the endogenous control should be expressed at roughly the same level as the RNA under study)." (See, Office Action, page 10.)

Applicants have amended independent Claims 1-4 to further distinguish the present invention. In particular, the claims have been amended to specify that the two or more target nucleic acids to be amplified are included in the reaction mixture that is heated in step (A). These claims have also been amended to recite that the copy number of each target nucleic acid in the reaction mixture in step (A) is not more than 10-fold different than the copy number of any other target nucleic acid in the reaction mixture. Support for this amendment may be found at page 8, lines 16-18, and in the preamble of original Claims 1-4.

Also, Claims 1-4 have been amended to specify that the reaction mixture includes "hot start DNA polymerase" (as disclosed for example in the '998 patent), which polymerase is "activated at T_1" of the amplification reaction. Support for this amendment may be found in the specification on page 27, lines 6-9, and U.S. Pat. No. 6,183,998 incorporated by reference therein.

Finally, Claims 1-4 have been amended to include as either an intermediate or final step to determine the efficiency of the PCR coamplification reaction, the step of detecting the two or more coamplification products, which amplification products have different sequence compositions. Support for this amendment may be found in the specification beginning on page 19, line 26, to page 21, line 25.

No new matter has been added by the above-referenced amendments to Claims 1-4. Entry of the amendments and allowance of the claims are respectfully requested.

In the present specification, Applicants have demonstrated that the use together of a hot start DNA polymerase and a volume exclusion agent, such as dextran or polyethylene glycol

(PEG), significantly improves the coamplification of two or more target nucleic acids in a multiplex PCR reaction. Further, the disclosed method is particularly advantageous where the copy number of each of the target nucleic acids at the start of the coamplification reaction is comparable, i.e., not more than 10-fold different than the copy number of any other target nucleic acid in the same reaction mixture. As shown in the Examples section (discussed below), this is in comparison to the coamplification of two or more target nucleic acids in a PCR reaction with either a standard (non hot-start) DNA polymerase and a volume exclusion agent or hot start DNA polymerase and no volume exclusion agent.

Examples 1 and 2 of the present specification, beginning on page 26, describe the coamplification of multiple target nucleic acids in the same PCR reaction, i.e., the PKC, SLP-65, ILGFMAR, c-fos, N-ras, fas, CD19 and CD5 murine genomic loci, all of which vary in size and sequence, using primers specific for each loci. Being genomic loci, the copy numbers of these targets were comparable. Amplification reactions were carried out with either (non hot-start) Taq DNA polymerase or hot start DNA polymerase (HotStartTaq®) and various concentrations (0%-6%) of either dextran or polyethylene glycol (PEG) as a volume exclusion agent.

As seen in the results shown in Table 2 on pages 28-29, the amplification reaction carried out with hot start DNA polymerase in the presence of either 1%, 3%, or 6% dextran showed up to a 6-fold increase in PCR product yield as compared with hot start DNA polymerase with 0% dextran. In contrast, also as seen on page 29, the same reaction performed with standard Taq DNA polymerase yielded, for the most part, no detectable PCR product regardless of the concentration of dextran in the amplification reaction mixture.

Similar results are seen in Table 3 on page 30, where the same reactions described above were performed in the presence of either 1%, 3%, or 6% PEG. Similar to the results shown in Table 2, the presence of hot start DNA polymerase and PEG yielded up to a 12-fold increase in PCR product as compared to the amplification reaction containing hot start DNA polymerase and 0% PEG. Also, similar to the results shown in Table 2, the amplification reaction carried out in the presence of standard Taq DNA polymerase yielded almost no PCR product regardless of the concentration of PEG in the amplification reaction.

Therefore, the results of the present specification clearly demonstrate that a multiplex PCR coamplification reaction containing multiple target nucleic acids, each present in the reaction mixture at a concentration that is no more than 10-fold different than the concentration of any other target nucleic acid in the reaction, shows a significant improvement in yield of final

product when the amplification reaction is performed in the presence of hot start DNA polymerase in combination with from 1% to 6% of a volume exclusion agent, as compared to the reaction carried out in the presence of hot start DNA polymerase and 0% volume exclusion agent or, surprisingly, as compared to the same amplification reaction carried out in the presence of standard Taq DNA polymerase and regardless of the concentration of volume exclusion agent.

Applicants assert that the claims of the present application are clearly distinguishable from the teachings of the Backus reference. There is no teaching in Backus to combine a volume exclusion agent and hot start polymerase for the coamplification of two or more target nucleic acids present at comparable, i.e., less than 10-fold different, copy numbers as disclosed in the present specification.

For instance, Examples 1 and 2 as described above also demonstrate that a multiplex PCR reaction using the hot start DNA polymerase described in the present invention, allows for a much lower concentration of PEG 8000 in the reaction mixture than disclosed in Backus, and achieves comparable or significantly better levels of amplification of PCR products with close to half the percentage or less of PEG in the reaction than disclosed by Backus. Examples 1-4 of Backus disclose a PCR reaction having one population of low copy nucleic acid (proviral HIV I DNA) and one population of hi copy nucleic acid (human β-globin DNA) amplified in the presence of 10% PEG. As seen in Table I of Backus, the best level of PCR amplification in the presence of PEG 8000 only resulted in a 3.4 fold increase over control levels. (Compare, Example 1 of Backus, 0.1μM primer level, **4.25** dye signal, with Control A (0.1 μM primer level, **1.25** dye signal)). In contrast, the present application demonstrates that in a multiplex PCR reaction having up to 8 different populations of nucleic acids, resulted in an over 4-fold increase in at least one of the populations with as little as 1% PEG and up to 12-fold increase in at least one population of target nucleic acids with only 6% PEG.

In addition, Applicants assert that the results presented in Backus actually teach away from the surprising results shown in the present application. Despite the teachings of Backus, Qiagen's results, in contrast, demonstrate that the combination of hot start DNA polymerase and dextran in a multiplex PCR reaction is not only efficient, but it is surprising (in light of Backus) that the reaction works at all.

The Examiner's attention is again directed to Table 2 on page 28 of the present application. Table 2 shows the results of the multiplex PCR reaction described above using hot start DNA polymerase and either 1%, 3%, or 6% dextran to coamplify targets of comparable

copy numbers. As seen in Table 2, in some instances, as little as 1% dextran present in the reaction mixture results in an over 2-fold increase in PCR product (as compared to 0% dextran control) and an over 6-fold increase in PCR product with as little as 6% dextran present in the reaction.

This is in contrast to Example 5 of Backus which shows the results of a PCR reaction in the presence of from 1%-10% dextran or from 1%-10% PEG. As seen in Table II of Backus, the presence of a volume exclusion agent, i.e., dextran, totally inhibits the PCR amplification reaction. According to Backus,

> "It is clear that . . . the presence of dextran sulfate **totally inhibits PCR**." (See, Backus, column 18, lines 5-10.) (emphasis added.)

In addition, Table II of Backus once again demonstrates the superior results the present invention displays over this reference with the use of PEG in combination with a hot start DNA polymerase. As seen in Table II, again the most improved results using PEG in the method disclosed by Backus still only results in a 2-fold improvement of amplification as compared with control (compare control (dye signal = **4.5**) with 6% PEG (dye signal = **9**)).

Therefore, even though Backus discloses that an antibody blocked DNA polymerase is suitable for use in the disclosed method (involving unequal targets), there is no teaching that the co-use of a volume exclusion agent with hot start DNA polymerase would lead to the unexpected results for coamplification of comparable targets shown in the Qiagen examples described above.

With respect to the Bustin reference cited by the Examiner as a secondary reference in light of Backus, in contrast to the Examiner's assertion, this reference does not relate to or disclose "the coamplification of two or more target nucleic acids present at comparable copy numbers". The Bustin reference is a review article describing methods for quantifying mRNA gene transcripts by reverse transcription polymerase chain reaction (RT-PCR). According to Bustin, RT-PCR-specific errors in the quantification of mRNA transcripts are compounded by variation in the amount of starting material, particulary when samples are taken from different individuals. The section of Bustin referred to by the Examiner concerns the normalization, i.e. calibration, to account for the errors that occur as a result of variations in the amount of mRNA starting material in RT-PCR reactions when quantifying mRNA transcripts again, particularly when comparing amplification of transcripts from samples taken from different individuals. According to Bustin, a common method for minimizing these quantification errors between

samples is to include a cellular RNA in the reaction, one that is expressed at a constant level in various tissues and, if possible, expressed at the same level endogenously as the mRNA gene transcript under study. Therefore, Bustin relates to normalization of an RT-PCR reaction using an RNA with known *in vivo* expression levels. Bustin does not, as asserted by the Examiner, "teach the coamplification of nucleic acids which are present at comparable copy numbers, wherein the maximum difference between the lowest and highest copy number is 10-fold". (See, Office Action, page 10.) Bustin discloses the use of a known internal standard to set a benchmark for normalizing unknown variations between separate amplification reactions.

Therefore, the combination of Backus taken with Bustin, does not render obvious the coamplification method of the present invention.

35 U.S.C. §103

The Examiner has rejected Claim 15 under 35 U.S.C. §103 as being unpatentable over Backus (*supra*), in view of Bustin (*supra*), and further in view of Mauzac et al., U.S. Pat. No. 4,740,594, Lantz et al., *Journal of Microbiological Methods*, 28: 159-167 (1997), as evidenced by JBC Handbook, 4th ed. (1997), entry on Dextran 40.

Claim 15, which depends from Claims 1, 2, 3, and 4, is directed to a particular molecular weight range for dextran that is suitable for use in the method of coamplification disclosed in Claims 1-4, specifically 40,000 to 60,000 daltons. According to the Examiner,

> "Backus does not explicitly disclose the use of dextran within the molecular weight range of 40,000 to 60,000 . . . Lantz teaches an embodiment of claim 12, characterized in that the dextran has a molecular weight in the range of 40,000 to 60,000." (See, Office Action, page 11.)
>
> * * *
>
> "It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to have modified the teachings of Backus and Bustin to incorporate the specific molecular weight of dextran disclosed by Lantz and Mauzac to arrive at the claimed invention . . ." (See, Office Action, page 12.)

As stated above, Backus actually teaches away from the present invention in that the use of dextran in the PCR reaction disclosed therein completely inhibited PCR at all concentrations of dextran tested. The Lantz et al. reference is directed to a method for removing PCR inhibitors from fecal samples prior to the amplification reaction.

> "Faecal specimens were used as a PCR-inhibitory samples . . . to demonstrate the general use of the aqueous two-phase system as a *sample preparation method prior to PCR*." (See, page 160, left column, second paragraph.) (emphasis added.)
>
> * * *
>
> "In the present study, we have investigated in more detail the efficiency of an aqueous two-phase system, composed of 8% (w/w) PEG 4000 and 11% (w/w) dextran 40, in removing PCR inhibitors." (See, page 160, left column, second paragraph.)

Specifically, Lantz et al. discovered that the dextran phase of the aqueous two-phase system bound the PCR inhibitors and allowed them to be removed before performing the PCR reaction.

> "Thus, the observed effect of dextran on PCR inhibition was assumed to be caused by binding or inactivation of PCR inhibitors originating from the faeces." (See, page 166, right column.)

Therefore, Lantz et al. disclose the use of dextran in combination with PEG to create an aqueous two-phase system for removal of PCR inhibitors prior to carrying out the PCR reaction and not as a component for improving the efficiency of the PCR reaction itself. Lantz et al. does not teach or discuss the use of hot start Taq polymerase in combination with dextran for improving the product yield of a multiplex PCR reaction.

The Mauzac et al. and the JBC Handbook references cited by the Examiner are related to the known anticoagulant properties of dextran or their derivatives and make no mention of either its use as a volume exclusion agent or its use as a volume exclusion agent in PCR.

For the reasons set forth above, entry of the amendments and allowance of Claims 1-16, 23, and 24 are respectfully requested.

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February 9, 2007
date

Nasim G. Memon

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 December 2001 (06.12.2001)

PCT

(10) International Publication Number
WO 01/92569 A2

(51) International Patent Classification[7]: C12Q 1/68

(21) International Application Number: PCT/GB01/02265

(22) International Filing Date: 23 May 2001 (23.05.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0013043.5 31 May 2000 (31.05.2000) GB
0013863.6 7 June 2000 (07.06.2000) GB

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/92569 A2

(54) Title: FORMULATION FOR POLYMERASE CHAIN REACTION AND VESSEL CONTAINING SAME

(57) Abstract: A formulation for use in effecting a Polymerase Chain Reaction, a dried composition of reagents including reaction buffer, dNTPs, at least two primers and a polymerase. The formulation is re-hydratable to be capable of effecting amplification of a target nucleic acid sequence of interest. The formulation incorporates a fluorescent reporter molecule capable of reporting by homologous detection the presence of amplified nucleic acid produced by the Polymerase Chain Reaction.

FORMULATION FOR POLYMERASE CHAIN REACTION AND VESSEL CONTAINING SAME

The present invention relates to formulations for use in effecting a Polymerase Chain Reaction and also to vessels containing such a formulation and which are intended for use in conducting such a reaction.

The Polymerase Chain Reaction (PCR) is one of the key tools in field of DNA diagnostics and enables the rapid and specific amplification of very small amounts of DNA sequences of interest. PCR is routinely performed either to obtain sufficient DNA for subsequent manipulation (e.g. for DNA sequencing or sub-cloning) or to identify the presence/absence of a specific nucleotide sequence in a large background of non-specific sequences.

Whilst PCR has revolutionised molecular biology, its ability to generate massive numbers of molecules of a particular DNA sequence from very few initial molecules can be a handicap in certain diagnostic situations. This is because of the ease of contaminating a PCR with the products of a previous reaction. Once contamination of a work area has arisen it can be very difficult to eradicate. This has lead to the adoption of complex and expensive methods of minimising this risk, such as purpose built laboratories, or laboratory areas, enzymatic pre- or post- PCR treatment of samples and controlling the use of such items as pipettes, lab coats etc, within defined work areas.

The PCR reaction itself generally consists of a number of preparative steps including the addition of a buffer solution, dNTP mix, primer solutions and usually a separate $MgCl_2$ solution followed by the addition of target and DNA polymerase. Many of the reagents can be included in a "master mix" that is then dispensed singly to individual reactions. Other reagents, usually the target and polymerase enzyme must be added individually to reaction tubes, which involves pipetting very low volumes (sub to

low μL) which can lead to considerable reaction variability. The complexity of the steps involved in optimising the performing PCR in this fashion requires a high degree of expertise in those entrusted with its performance and constant vigilance and monitoring of contamination issues.

A proposal to overcome the disadvantages set out in the previous paragraph is set out in US-A-5 861 251 (Park et al) which discloses a ready-to-use PCR reagent formulation obtained by lyophilisation of an aqueous mixture comprising a reaction a DNA polymerase, buffer, $MgCl_2$, dNTPs, PCR primers, glucose or glucitol as a stabilising and sedimenting agent, and a water soluble dye selected from bromphenol blue, xylene cyanol, bromocresol red and cresol red. This lyophilised mixture has the advantage that it simplifies the multi-step PCR manipulation in that all components (except target) for effecting amplification are included in the pre-prepared mixture such that all that is required is addition of an aqueous sample containing (or potentially containing) the target. Furthermore, the water soluble dye facilitates identification of complete mixing of the PCR reagent and test sample and saves the trouble of adding a sample loading buffer which is otherwise required for analysis of PCR products. As a result, the formulations of US-A-5 861 251 provide the advantage of avoiding carry-over contamination into the PCR reaction mix. However detection of the amplified product is effected by running the product mixture on a gel. This necessities opening of the tube, to apply the product mixture to the gel, thus once again giving rise to the possibility of cross-contamination.

It is an object of the present invention to obviate or mitigate the above-mentioned disadvantages.

According to a first aspect of the present invention there is provided a formulation for use in effecting a Polymerase Chain Reaction, the formulation comprising a dried composition of reagents including reaction buffer, dNTPs, at least two primers and a

polymerase and said formulation being re-hydratable to be capable of effecting amplification of a target nucleic acid sequence of interest characterised in that the formulation incorporates a fluorescent reporter molecule capable of reporting by homologous detection the presence of amplified nucleic acid produced by the Polymerase Chain Reaction..

As with that proposed in US-A-5 861 251, the formulation of the invention is such that only a single addition of aqueous target sample to the formulation is required to produce an aqueous reaction mixture containing all necessary components for PCR amplification of target nucleic acid sequence.

The formulation of the invention does however have the significant additional advantage that the presence, in the formulation, of the fluorescent reporter molecule means homologous detection may be used. Thus the progress of the reaction may be followed by real-time detection techniques avoiding the need for post-reaction manipulation of the product mixture (e.g. transferring the mixture to a gel, or even opening a vessel in which the product mixture is contained) thereby avoiding any possibility of cross-contamination. This has dramatic consequences for the set-up of laboratories that PERform PCR-based diagnostic reactions as, currently, extreme care has to be taken during the performance of the reaction to prevent cross-contamination. Using the present invention, no particular contamination controls would be needed other than those routine in a molecular biology laboratory. There are also additional benefits, including having much more defined reaction conditions (as essentially all the reactants could come from the same batch, convenience, longer shelf life etc).

The invention also provides, according to a second aspect thereof, a vessel (e.g. a reaction tube) containing a pre-measured amount of the formulation of the invention.

The vessels (with premeasured formulation) may be provided with a suitable closure element and supplied to end users who, after removal of the closure element merely, need only to add the aqueous sample and then re-close the vessel. The end user may be a person in a laboratory where the PCR reaction is then effected. Alternatively the end user may be out "on-site" collecting samples which can then be added to the vessel as soon as collected, the vessel then being sent to a laboratory for conducting the PCR reaction.

Conveniently, after addition of the aqueous sample to the vessel the latter is closed with a non-removable closure element so that there can be no possible interference with the PCR reaction which, as described above, may be effected and monitored without the need to remove the closure element. For this purpose, the inner surface of the vessel (adjacent the mouth thereof) and the outer surface of the closure element may be provided with inter-engageable formations allowing insertion of the closure element into the vessel but preventing withdrawal therefrom. These inter-engagable formations should be positioned such that the closure element is capable of being removable provided that it has not been inserted into the vessel beyond a certain degree. As such, the dried composition may be incorporated into the vessel and the closure element removably applied thereto. Subsequently the closure element may be removed to permit addition of the sample and then subsequently inserted sufficiently far into the vessel so that it becomes non-removable.

The formulation of the invention may be prepared by lyophilisation of an aqueous solution of the required components, e.g. by lyophilisation using the procedures disclosed in US-A-5 861 251. Preferably the solution includes a stabiliser which may for example be glucose, glucitol or trehalose.

The dried formulation of the invention may be such that, per ml of reconstituted reaction medium, it comprises:

Component	Amount
Polymerase	0.01-0.04 units
Primers	0.1-10 pmoles
Fluorescent Reporter	0.1-10 pmoles
KCl	10-100 nmoles
Tris-HCl	10-20 nmoles
Triton X100	0.5-5mg
$MgCl_2$	0.5-10 nmoles
DNTP(each)	50-500 pmoles
Stabiliser (e.g. trehalose)	0.1-15% w/w

Once the lyophilised sample has been rehydrated by addition of aqueous sample, the PCR reaction may be conducted by procedures well known in the art, e.g. using thermal cycling.

The fluorescent reporter molecule included in the formulation of the invention may for example be one which reports a change in the amount of double stranded DNA present in the reaction, e.g. an intercalating dye such as Ethidium Bromide, CyBr Green or PicoGreen. Alternatively the fluorescent reporter molecule may be one which works in conjunction with a quencher moiety so as to be capable of reporting on the presence of specific nucleotide sequences in the mixture and may, for example, be a TaqMan probe, Molecular Beacon, Sunrise primer and Scorpion primer (Registered Trade Marks).

The polymerase may be a DNA polymerase and may be a thermally stable polymerase, e.g. Taq polymerase. There is however a deficiency with Taq in that it becomes active (though at low efficiency) at relatively low temperatures. As a result, mis-primed reactions can occur at these low temperatures before the PCR begins. Thus,

re-hydration of the reaction mix, if not carried out at or above the annealing temperature of the primers, could lead to non-specific reactions occurring that will reduce the efficiency of the PCR and could lead to false positive reactions being reported. It is therefore preferred that the polymerase in the formulation of the invention is a "hot-start" polymerase. "Hot-start" polymerases are known in the art and are such that a heating step is required to activate the polymerase (which has typically been inactivated with an antibody). The "hot-start" polymerase should be one for which the "means" of inactivation of the enzyme (e.g. an antibody) must be able to withstand the drying/rehydration procedure. The advantage that the use of such an enzyme confers is that the re-hydration of the dried reagent composition can occur at ambient temperature without initiating potentially ruinous side reactions prior to heating the sample and cooling to annealing temperature at which only desired reactions can occur.

CLAIMS

1. A formulation for use in effecting a Polymerase Chain Reaction, the formulation comprising a dried composition of reagents including reaction buffer, dNTPs, at least two primers and a polymerase and said formulation being re-hydratable to be capable of effecting amplification of a target nucleic acid sequence of interest characterised in that the formulation incorporates a fluorescent reporter molecule capable of reporting by homologous detection the presence of amplified nucleic acid produced by the Polymerase Chain Reaction..

2. A formulation as claimed in claim 1 wherein the fluorescent reporter molecule is capable of reporting a change in the amount of double stranded DNA in the Polymerase Chain Reaction.

3. A formulation as claimed in claim 2 wherein the fluorescent reporter molecule is an intercalating dye.

4. A formulation as claimed in claim 3 wherein the intercalating dye is selected from Ethidium Bromide, CyBr Green and PicoGreen

5. A formulation as claimed in claim 1 wherein the fluorescent reporter molecule is one which works in conjunction with a quencher moiety so as to be capable of reporting the presence of specific nucleotide sequences in the mixture.

6. A formulation as claimed in claim 1 to 5 wherein the polymerase is a DNA polymerase.

7. A formulation as claimed in claim 6 wherein the polymerase is a thermally stable polymerase.

8. A formulation as claimed in claim 7 wherein the thermally stable polymerase is Taq.

9. A formulation as claimed in claim 6 or 7 wherein the polymerase is a "hot-start" polymerase.

10. A vessel for conducting a polymerase chain reaction therein, said vessel containing a pre-measured amount of the formulation as defined in any one of claims 1 to 9.

11. A vessel as claimed in claim 10 which is a reaction tube.

12. The combination of a vessel as claimed in any one of claims 10 and 11 and a closure element for closing the vessel after the addition of aqueous sample thereto.

13. The combination as claimed in claim 12 wherein the closure element is capable of effecting irreversible closure of the vessel.

14. The combination as claimed in claim 13 wherein the inner surface of the vessel (adjacent the mouth thereof) and the outer surface of the closure element are provided with inter-engageable formations allowing insertion of the closure element into the vessel but preventing withdrawal therefrom.

15. A method of effecting a polymerase chain reaction comprising adding an aqueous sample potentially containing target nucleic acid sequence to a vessel as claimed in claim 10 or 11 or the vessel of the combination as claimed in claim 12 or 13, effecting the polymerase chain reaction, and effecting fluorescence detection of the product mixture in situ in the vessel.

16. A method as claimed in claim 15 when used with the combination of claim 12 or 13, the method additionally comprising the step of inserting the closure element into the vessel after addition of the sample and prior to effecting the polymerase chain reaction.

17. A method as claimed in claim 16 when used with the combination of claim 14 wherein the step of inserting the closure element into the vessel effects irreversible closure.

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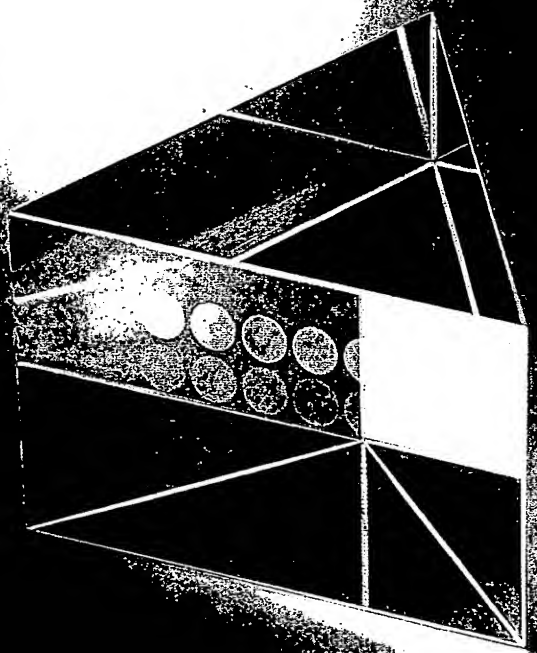

HotStarTaq™ DNA Polymerase

HotStarTaq™ DNA Polymerase, a modified form of QIAGEN® Taq DNA Polymerase, provides high specificity in hot-start PCR.

Features and benefits

- Higher PCR specificity
- Reduced nonspecific amplification
- Easy handling

Product Format Options

Components	HotStarTaq DNA Polymerase	HotStarTaq Master Mix Kit (premixed solution)*
HotStarTaq DNA Polymerase	✓	✓
PCR Buffer†	✓	✓
Q-Solution	✓	–
dNTP Mix‡	–	✓
$MgCl_2$ solution	✓	✓
Distilled water	–	✓

** HotStarTaq Master Mix is a premixed solution containing HotStarTaq DNA Polymerase, PCR Buffer, and dNTPs. The solution provides a final concentration of 1.5 mM $MgCl_2$ and 200 µM each dNTP.*

† Provides a final concentration of 1.5 mM $MgCl_2$

‡ Provides a final concentration of 200 µM each dNTP

Principle

HotStarTaq DNA Polymerase, a modified form of QIAGEN *Taq* DNA Polymerase, is supplied in an inactive state that has no polymerase activity at ambient temperatures. This prevents extension of nonspecifically annealed primers and primer–dimers formed at low temperatures during PCR setup and the initial PCR cycle (Figures 2.7 and 2.8, Table 2.2). HotStarTaq DNA Polymerase is activated by a 15-minute incubation at 95°C which can be incorporated into any existing thermal-cycler program. Every lot of HotStarTaq DNA Polymerase is subjected to a comprehensive range of quality control tests, including a stringent PCR specificity and reproducibility assay in which low-copy targets are amplified.

Table 2.2 Comparison of hot-start methods

	HotStarTaq DNA Polymerase	Hot-start enzyme from Supplier A	Antibody-mediated	Manual	Wax barrier
Specific amplification	++	+	+	+/–	+/–
Minimal PCR optimization	++	+/–	+/–	–	–
Easy to use	++	++	+	–	–

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